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Potential Diagnostic Biomarkers for Human Uterine Mesenchymal Tumours: Especially LMP2/β1i and Cyclin E1-Differential Expressions

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ABSTRACT

Aims: Although the majority of smooth muscle neoplasms found in the uterus are benign, uterine leiomyosarcoma is extremely malignant, with high rates of recurrence and metastasis. The development of gynecologic tumors is often correlated with secretion of female hormone; however, the development of human uterine leiomyosarcoma is not substantially correlated with hormonal conditions, and the risk factors are unclearly understood. Importantly, a diagnostic-biomarker, which distinguishes malignant human uterine leiomyosarcoma from benign tumor leiomyoma is yet to be established. It is necessary to analyze risk factors associated with human uterine leiomyosarcoma, in order to establish a diagnostic-biomarker and a clinical treatment method. **Methodology**: Histology and Immunofluorescence Staining: tissue sections (5 μm) were prepared and stained with H&E for routine histological examination or were processed further for immunofluorescence staining with appropriate antibodies. Furthermore, a total of 57 patients between 32 and 83 years of age and diagnosed as having smooth muscle tumors of the uterus were selected from pathological files. Immunohistochemistry staining for LMP2/β1i and cyclin E1 was performed on serial human uterine leiomyosarcoma, leiomyoma and myometrium sections. **Results**: Homozygous deficient mice for a proteasome subunit LMP2/β1i spontaneously develop uterine leiomyosarcoma, with a disease prevalence of ~40% by 14 months of age. Defective LMP2/β1i and cyclin E1 positive expressions in human uterine leiomyosarcoma were demonstrated, but the reverse result was obtained in human leiomyoma and myometrium. **Conclusions**: LMP2/β1i and cyclin E1 differential expressions may be one of the risk factors for human uterine leiomyosarcoma. LMP2/β1i and cyclin E1 may be potential diagnostic-biomarker and targeted-molecule for a new therapeutic approach.

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KEYWORDS: *LMP2/β1i; cyclin B1; uterine leiomyosarcoma; diagnostic-biomarker*

INTRODUCTION

The uterus, the organ in which the embryo grows, is composed of three layers, the uterine endometrium which serves as a bed for the embryo; the myometrium of the wall which protects the embryo; and a serous membrane enveloping the uterus. In general, the term uterine tumor refers to an epithelial malignant tumor of the uterus, which is roughly classified as a tumor of the uterine cervix or the uterine body. Because of the prevalence of medical checkup, the rate of mortality from uterine cervix cancer is decreasing, and usually detected at a very early stage. In contrast, the mortality rate for cancer of the uterine body is increasing, and the disease is rarely detected at the initial stages. While most tumors of the uterine body are adenocarcinomas (derived from the subintimal gland), the uterine

cervix tumors are classified into squamous cancer and adenocarcinoma. Smooth muscle tumors (SMTs) which develop in the myometrium have been traditionally divided into benign leiomyoma (LMA) and malignant uterine leiomyosarcoma (Ut-LMS) based on cytological atypia, mitotic activity and other criteria. Ut-LMS is relatively rare, having an estimated annual incidence of 0.64 per 100,000 women (1). Ut-LMS accounts for 2% to 5% of tumors of the uterine body and develops more often in the muscle layer of the uterine body than in the uterine cervix. As Ut-LMS is resistant to chemotherapy and radiotherapy, surgical intervention is virtually the only means of treatment (2). The prognosis for Ut-LMS is not good, and the five-year survival rate is approximately 35% (3,4). However, developing an efficient adjuvant therapy is expected to improve prognosis for Ut-LMS. LMA may occur in as many as 70%~80% of women by the age of 50 years (FACT SHEET-Uterine Fibroids. 2010). Distinguishing LMA from Ut-LMS is very difficult, and a diagnosis generally requires surgery and cytoscopy (5). Diagnostic categories for uterine SMTs and morphological criteria are used to assign cases (6,7) (NOTE 1). The non-standard subtypes of uterine SMTs such as the epithelioid and myxoid types are classified in a different way using these features, so the establishment of a diagnostic method for the identification of non-standard smooth muscle differentiation is important.

The molecular mechanisms by which LMA and Ut-LMS develop are not yet known, though tumors that have developed in the myometrium for some reason gradually become larger due to the influence of the female hormone, estrogen or somatic mutation of MEDIATOR COMPLEX SUBUNIT 12 (MED12), and generate tumors (8). However, no correlation between the development of Ut-LMS and hormonal conditions, and no obvious risk factors have been found.

Although cases accompanied by hypocalcaemia or eosinophilia have been reported, neither clinical abnormality is an initial risk factor for Ut-LMS. The identification of a risk factor associated with the development of Ut-LMS would contribute to the development of preventive and therapeutic treatments.

Cytoplasmic proteins are mostly degraded by a protease complex, which has many substrates consisting of twenty-eight 20 to 30-kDa subunits, referred to as the 20S proteasome (9). The proteasomal degradation is essential for many cellular processes, including the cell cycle, the regulation of gene expression and immunological function. Interferon (IFN)-γ induces the expression of large numbers of responsive genes, proteasome subunits, i.e., low-molecular mass polypeptide (LMP)2/β1i, LMP7/β5i, and LMP10/β2i. A molecular approach to studying the correlation of IFN-γ with tumor cell growth has drawn attention (10). Homozygous mice deficient in LMP2/β1i show tissue- and substrate-dependent abnormalities in the biological functions of the proteasome, and LMP2/β1i correlates to cell survival (11,12). Ut-LMS occurred in female LMP2/β1i-deficient mice at age 6 months or older, and the incidence at 14 months of age was about 40% (13,14). The curve indicating the incidence in mice is similar to that indicating the incidence of human Ut-LMS, which occurs after menopause.

Advanvce in research on the cell cycle have revealed that interaction between cyclins, cyclin-dependent kinases (cdks), and tumor suppressor gene products play an essential part in cell cycle progression (15). Cyclins, which form complexes with cdks, are a group of proteins periodically expressed during the cell cycle (16). While normal cells are generally thought to have a normal cell cycle regulatory system, a deranged expression of these cell cycle-related factors appear to be involved in the malignant transformation of cells (17). We focused cyclin E1 expression patterns in human uterine mesenchymal tumors, because cyclin B plays in integral role in many types of cancer (18,19,20,21). Hyperplasia (uncontrolled cell growth) is one of the hallmarks of cancer. Because cyclin B is necessary for cells to enter mitosis (M), and therefore necessary for cell division, cyclin B levels are often de-regulated in tumors. When cyclin E1 levels are elevated, cells can enter M phase prematurely and strict control over cell division is lost, which is a favorable condition for cancer development. This being so, the present study was undertaken to investigate the expression of cyclins, especially cyclin E1/mitotic cyclin, which is necessary for the progression of the cells into and out of M phase of the tumor cell cycle, in uterine SMTs using immunohistochemical (IHC) and western blotting (W.B.) experiments. Here in genetic analysis, we identify LMP2/β1i and cyclin E1 deferential expressions in human uterine mesenchymal tumors. LMP2/β1i and cyclin E1 may be a potential diagnostic-biomarker and targeted-molecule for a new therapeutic approach.

Materials and methods

Tissue collection. A total of 57 patients aged between 32 and 83 years who were diagnosed as having smooth muscle tumors of the uterus were selected from pathological files (22,23). Serial sections were obtained from at least 2 tissue blocks from each

patient for hematoxylin and eosin staining and immunostaining. All tissues were used with the approval of the Ethical Committee of Shinshu University after obtaining written consent from each patient.

Immunohistochemistry (IHC). IHC staining for LMP2/β1i and cyclin E1 was performed on serial human Ut-LMS or LMA sections. Antibody for cyclin E1 were purchased from Immunotech (Marseille, France). The LMP2/β1i antibody was produced by SIGMA-Aldrich Israel Ltd. (Rehovot, Israel). IHC was performed using the avidin-biotin complex method as described previously. Briefly, one representative 5-μm tissue section was cut from a paraffin-embedded sample of a radical hysterectomy specimen from each patient with Ut-LMS. Sections obtained from patients were deparaffinized and rehydrated in graded concentrations of alcohol, incubated with normal mouse serum for 20 min, and then incubated at room temperature for 1 h with primary antibody. Afterwards, sections were incubated with a biotinylated secondary antibody (Dako, CA) and then exposed to a streptavidin complex (Dako). The completed reaction was revealed by 3, 3′-diaminobenzidine, and the slide was counterstained with hematoxylin. Normal myometrium portions in the specimens were used as positive controls. Negative controls consisted of tissue sections incubated with normal rabbit IgG instead of the primary antibody. These experiments were registered at Shinshu University in accordance with local guidelines (approval no. M192).

Results

In general, it is not easy to distinguish human LMA from Ut-LMS, however, in mice, because of such characteristic pathological findings, significant weight loss, and skeletal muscle metastasis, a tumor that develops in the uterus of an LMP2/β1i-deficient mouse can be considered malignant, i.e., Ut-LMS. The IHC studies with human tissue samples revealed a serious loss in the ability to induce LMP2/β1i expression in human Ut-LMS tissue in comparison with LMA or normal myometrium located in the same section. Of the 32 cases we examined with Ut-LMS, 29 cases were negative for LMP2/β1i expression, 1 case was focally positive, and 1 case was partially positive. One Ut-LMS case was stained for LMP2/β1i. Cyclin E1 was furthermore the focus of the present study because of the high ratio of cyclin B1 expression in Ut-LMS compared with LMA and myometrium. In IHC studies, all Ut-LMS cases were stained for cyclin E1. Pathological examination of surgical samples showed the presence of a mass measuring 3 cm in its largest diameter in the lumbar quadrate muscle without a fibrous capsule. All lymph nodes were negative for Ut-LMS metastases, and IHC analyses showed positivity for cyclin E1 and Ki-67 and negativity for LMP2/β1i. Histological findings were consistent with metastatic LMS for the skeletal muscle and rectum lesions.

Although we have previously demonstrated that the abnormal expression of the ovarian steroid receptors, Tumor Protein 53 (TP53) and MARKER OF PROLIFERATION KI67 (Ki-67) and mutations of TP53 were frequently associated with Ut-LMS, defective LMP2/β1i expression appears to be more characteristic of Ut-LMS than these factors (24,25) (Table). In further experiments, almost all LMA showed staining for both Estrogen Receptor (ER) and Progesterone Receptor (PR) irrespective of the phase of the menstrual cycle, and the number of Ki-67 positive cells in LMA was significantly lower than that of Ut-LMS (Table). IHC staining also demonstrated that almost all LMA showed staining for TP53 (Table).

Discussion

A recent report showed the expression of *Lmp2/□1i* mRNA and protein in luminal and glandular epitheliua, placenta villi, trophoblastic shells, and arterial endothelial cells (26). These results implicate LMP2/β1i in the invasion of placental villi, degradation of the extracellular matrix, immune tolerance, glandular secretion, and angiogenesis (26). The present study should help to elucidate the regulatory role of LMP2/β1i in the implantation of embryos. Cyclin E1 immunoreactivity was observed in the nucleus and the cytoplasm in all the Ut-LMS cases examined, the other hand most cases of leiomyoma and the normal myometrium were negative for cyclin E1. Cyclin E1 is a regulatory protein involved in mitosis, the gene product complexes with Cdk1 to form the maturation-promoting factor (MPF) (27). Cyclin E1/Cdk1 is involved in the early events of mitosis such as chromosome condensation, nuclear envelope breakdown, and spindle pole assembly. If cyclin E1 levels are depleted the cyclin E1/Cdk1 complex cannot form, cells cannot enter M phase and cell division slows down. Some anti-cancer therapies have been designed to prevent cyclin E1/Cdk1 complex formation in cancer cells to slow or prevent cell division (28). Most of these methods have targeted the Cdk1 subunit, but there is an emerging interest in the oncology field to target cyclin E1 as well. That is, revelation control of cyclin E1 may become a clue to development of the new cure to uterine leiomyosarcoma. Clinical risk factors for its development however, have not been identified

because of the absence of a suitable animal model. The LMP2/β1i-deficient mouse was the first animal model of spontaneous Ut-LMS to be established. Defective LMP2/β1i expression may be one of the causes of Ut-LMS. To demonstrate whether LMP2/β1i and cyclin E1 are a potential biomarker for distinguishing Ut-LMS from LMA, we are investigating the reliability and characteristics of LMP2/β1i and cyclin E1 as a diagnostic indicator with several clinical research facilities. The clinical research is yet to be concluded, and large-scale clinical studies need to be performed.

In conclusion, clarification of the correlation between these factors "LMP2/β1i and cyclin E1" and the development of Ut-LMS, and therefore the identification of specific risk factors may lead to the development of new treatments for the disease. Ut-LMS is refractory to chemotherapy and has a poor prognosis. The molecular biological and cytological information obtained from further research experiments with human tissues and LMP2/□1i-deficient mice will contribute remarkably to the development of preventive methods, a potential diagnostic- biomarker, and new therapeutic approaches against Ut-LMS.

(NOTE 1) The typical gross appearance is a large (>10cm), poorly circumscribed mass with a soft, fleshy consistency and a variegated cut surface that is grey-yellow to pink, with foci of hemorrhage and necrosis (6,7). The histologic classification of uterine sarcomas is based upon homology to normal cell types and includes human Ut-LMS (analogous to myometrium), stromal sarcoma (analogous to endometrial stroma), and other heterologous cell types (i.e., osteosarcoma, liposarcoma). Microscopically, most human Ut-LMS are overtly malignant, with hypercellularity, coagulative tumor cell necrosis, abundant mitoses [>10 to 20 mitotic figures (mf) per 10 high power fields (hpf)], atypical mitoses, cytologic atypia, and infiltrative borders. The mitotic rate is the most important determinant of malignancy but is modified by the presence of necrosis and cytologic atypia. A diagnosis of human Ut-LMS may be made in the presence of tumor necrosis and any mitosis. In the absence of tumor necrosis, the diagnosis can be made with moderate to severe cytologic atypia and a mitotic index greater than 10mf/10hpf. Without tumour necrosis and significant atypia, a high mitotic index is compatible with a benign clinical course, however, data is limited (6,7).

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